Review Article

NMR Based Metabolomics Approach and Its Clinical Applications

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Abstract

Recognizing, understanding and elucidating the pathophysiological mechanism of any disease is of prime importance for clinical research. The ever-evolving omic technology "metabolomics" has the potential to detect quantitatively large number of metabolites in biological fluids or tissues in a single shot. It is a well-established technique for early detection, therapeutic response and to understand the pathophysiology of the disease. Metabolomics being close to phenotype is the global representation of all the upstream (genetic, transcriptomic, proteomic) changes that occur at metabolic level in an individual. Nuclear magnetic resonance (NMR) based metabolomics is an information rich analytical technique with a broad spectrum of applications. The advanced statistical and chemometric approaches help dealing with complex and voluminous data so generated and also provides a powerful platform for clinical as well as translational research. The present review focuses on clinical applications of NMR based metabolomics in various diseases such as cancer, diabetes, coronary artery diseases and celiac disease. The NMR based experimental approach and the various pre and post processing steps including predictive modelling, model verification and pathway analysis has also been discussed

Keywords: NMR based metabolomics, chemometrics, Biomarkers, Metabolic fingerprinting

Introduction

Metabolomics is an emerging technology that holds the potential to improve the understanding of biological system at the molecular level when combined with genomics and proteomics approach. Metabolome refers to the collection of the metabolites present within the organism, cells or tissues.[1] It is considered as the downstream of the genome, transcriptome and proteome and in principle is the resultant of all the upstream levels and also of environment interaction.[1,2] Metabolome of a living system comprises of both exogenous metabolites and endogenous metabolites. Endogenous metabolites are those that are synthesized within the body while exogenous metabolites such as drugs and nutrients are the one that are introduced into the biological system (cell) from outside.[3,4]

Metabolomics can be classified on the basis of analytical approach used as targeted and untargeted metabolomics.[5,6] Targeted metabolomics approach focuses on the quantitative analysis of specific metabolites involved in particular biochemical pathways, whereas untargeted metabolomics approach focuses on the complete metabolic profiling of biological specimens. Untargeted metabolomics approach measures as many metabolites as possible to compare between biological samples. The main analytical techniques employed in the metabolomics study are nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry. The two major aims of metabolomics of disease are: first, to provide valuable insight into the pathophysiology of the disease, and second, identification of biomarker/s. In recent years, metabolomics approach has been used to elucidate metabolic abnormalities associated with various human diseases like breast cancer,[7] prostate cancer,[8] coronary artery diseases,[9] inflammatory bowel diseases[10] and celiac disease[11] and identify biomarkers of diagnostic and prognostic value. In the present review we have presented the steps required for metabolomics data acquisition, analysis and the applications of NMR based metabolomics in various diseases.

Experimental Aspects of NMR Metabolomics

The major advantage of NMR spectroscopy is that analysis of different types of biological samples like cell/tissue extracts, and body fluids like cerebrospinal fluid, blood, urine as well as seminal fluid can be used. Most of the sequences used to study biological samples are combined with pre-saturation pulses. In pre-saturation, solvent signal (water) is suppressed by selectively irradiating with a weak radiofrequency field (60-70 dB on spectrometers) during the relaxation delay, which is usually of an order of 2-5 seconds. Longer relaxation delays are used for quantitation of the concentration of the metabolites. The selection of pulse sequences depends on the nature of biological sample and information required from that sample. In general, the 1D version of Nuclear Overhauser Enhancement Spectroscopy (NOESY) sequence is used for acquisition of proton NMR spectrum of urine samples, Carr Purcell Meiboom Gill (CPMG: serum) and single pulse for cell/tissue extracts. After the acquisition of data, the NMR spectrum is obtained by Fourier Transformation of the time domain data and is corrected for phase and baseline distortions. For untargeted metabolomics, each NMR spectrum is then reduced into regions of equal width (mostly 0.04 ppm) known as bins or buckets. These bins are then subjected to multivariate data analysis methods to build the statistical model for classification of groups. The statistically significant bins representing the metabolites are then integrated and the relative concentration or the absolute concentration can be calculated. In the targeted approach, the concentration or integration data of identified specific metabolites are used for performing statistical analysis. For identification and confirmation of metabolites, 2D experiments such as correlation spectroscopy (COSY) and total correlation spectroscopy (TOCSY), heteronuclear correlation experiments (HSQC, HMBC) are carried out. The quantitative analysis of the identified metabolites can be used to perform either the targeted or the untargeted metabolomics.[10,11] Figure 1 shows the flowchart describing the details of major steps involved in metabolomics approach using NMR spectroscopy techniques.

Analysis of Metabolomics data

Data analysis is a crucial step in metabolomics due to its high dimensionality and complexity. Usually, complete analysis of metabolomics data involves following steps: pre-treatment, pre-processing, processing, model validation and post-processing of the dataset.[12]

Pre-treatment of dataset

The initial step of metabolomics data analysis involves pre-treatment of dataset that resolves the heteroscedasticity of multiple variables in the dataset. Pre-treatment involves normalization of dataset that include centering. Scaling and transformation.[13-15] Centering adjusts the concentration differences between the metabolites by converting all measurements to vary around zero instead of mean of the metabolite. Usually mean centring is used for metabolomics data,[16] scaling removes the variation present in the concentration of the metabolites which can influence the data analysis. Numerous scaling procedures are available but mean centering, autoscaling (standard devia-

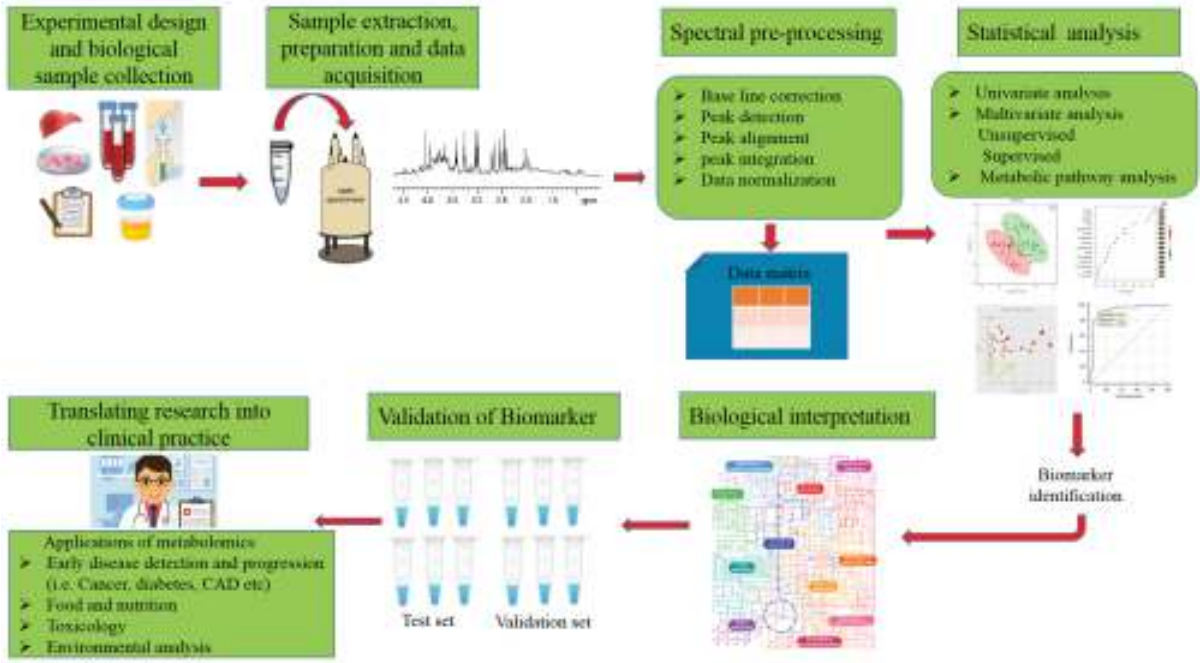

Figure 1: Workflow of targeted/untargeted NMR based metabolomics approach

tion), pareto scaling (square root of the standard deviation) are commonly used. Transformations are non-linear conversions that are applied to correct the heteroscedasticity of dataset, thereby reducing the variability between variables. Log transformation is mostly used for metabolomics data,[17] however, cubic root[18] and quantile[19] transformations can also be used.

Pre-processing

The aim of the pre-processing methods is to obtain an overview of the important variables prior to processing by prediction model. Two types of approaches are normally used univariate and multivariate analysis.

Univariate analysis

Univariate analysis examines, only one variable at a time. The commonly used methods for the parametric data are T-tests and analysis of variance (ANOVA), whereas, for non-parametric data, Mann-Whitney test (MW-test) and Wilcoxon test (W-test) are used. Additionally, univariate analysis also measures correlations between variables and response where Pearson's correlation is a preferred choice for parametric data while Spearman's correlation[20] is usually used in non-parametric datasets.

Multivariate analysis

Multivariate analysis includes unsupervised and supervised data analysis. In unsupervised method of data analysis, the modelling is based only on the explanatory variables, with no prior knowledge of the dataset. The most common are principal component analysis (PCA)[17] and hierarchical cluster analysis (HCA).[17] PCA linearly transforms the metabolic features into a set of linearly uncorrelated (orthogonal) variables into score vectors and loadings, called principal components.[21] PCA is also used to assess the data quality by identifying sample outliers present in the study. HCA is also a clustering and visualization tool based on variables similarities/dissimilarities and can be done in agglomerative mode (aggregation of samples into clusters) or divisive mode (division of complete dataset into clusters).[22,23]

Processing Methods

After variable selection, the next step is the building up of a predictive model to classify new samples, identify biomarkers and to explore the mechanisms of metabolomics studies (i.e. metabolic pathways). At this stage, the supervised methods play an important role and the widely used supervised method in metabolomics is partial least squares (PLS). It can be used either as regression analysis or as binary classifier. PLS maximizes the covariance between the variable of interest and metabolomics data, producing score vectors and loading vectors. The loading vector measures how much a feature contributes to demarcate between groups. In PLS, few metabolites that do not correlate with the variables present in the study and can influence the results, so in order to solve this problem, orthogonal PLS (OPLS) was developed.[24] OPLS is further expansion of PLS that includes an orthogonal signal correction. Support vector machines (SVM), random forests (RF) and logistic regression analysis (LRA) are other type of supervised analysis that use nonlinear methods to build classifiers based on metabolomics data.

Model validation

The performance and stability of the predictive models is a fundamental step for metabolomics data analysis and requires model validation. The aim of the validation process is to assess the potentiality of model so as to accurately classify the hypothesized association between variables and responses.[12] The coefficient of determination (R2) is the simplest method for choosing the optimal model structure and is indicated as the ratio between 0 and 1, where 1 indicates the perfect prediction. This validation method is used for small datasets, however, for high-dimensional and complex metabolomics datasets, cross validation (CV) methods are the choice of preference. In CV, the data is split into two sets, predictor variables (training set) and predictive model (validation set).[21] The mostly used CV method are k-fold, leave-one-out cross validation (LOOCV) and Monte Carlo cross validation (MCCV). However, the standard method used for model validation now days is the receiver operating characteristic (ROC) curve.[25] The predictive ability of the model is validated in terms of specificity (true negative rate) and sensitivity (true positive rate). The ROC curve is a plot of sensitivity versus (1 - specificity) with a series of cut-off points. ROC is summarised as a single matric known as area under curve (AUC). AUC quantitatively measures the ability of predictive model and is expressed as ratio between 0 and 1, with value near to 1 indicates perfect classifier.[25]

Post-processing

The goal of post-processing step is to identify a useful biomarker or panel of biomarkers that helps in accurate prediction of particular phenotype. Usually, metabolic pathway analysis is the most useful approach that provides a sketch of the relationship between different recognized metabolites and metabolic pathways and other biological networks. Pathifier[26] and metaboanalyst[18,11] are the most commonly used software for pathway analysis in metabolomics.

Applications of NMR based metabolomics in various diseases

Metabolomics approach has been applied to study and analyse a wide range of diseases, through the analysis of different kinds of biological samples, including urine, blood plasma/serum, cerebrospinal fluid (CSF), seminal fluid as well as intact tissue biopsies and tissue extracts. In addition to its application in early disease detection and progression such as (cancer,[27] neurological diseases[28,29] and diabetes[30]), metabolomics has varied research domains such as toxicology,[31] quality control (QC) of herbal extracts,[32] food and nutrition[33] and environmental analysis.[31]

Some of the NMR based metabolomics studies with reference to specific diseases are discussed below.

Cancer

NMR spectroscopy has been applied to understand the metabolic signatures of various types of cancer, such as breast cancer,[34,35] prostate cancer,[36,37] pancreatic cancer[38,39] and lung cancer.[40] The metabolome of cancer cells is well characterized by increased glycolytic and glutaminolytic activity and alteration in total choline (Cho) level.[41] However, different cancer cells are found to be distinct in their metabolism.[42]

Breast Cancer

The metabolic profile of perchloric extract of tumor and non-involved breast tissue was investigated using NMR based metabolomics. Lower levels of glucose (Glc) while higher levels of phosphocholine (PC) were observed in tumor tissue compared to normal breast tissue.[43] Proton (^{1}H) NMR spectroscopy was also employed to compare the metabolic profile of benign lesions, invasive breast cancer and carcinoma in situ.[44] The study revealed that Cho could be used for discrimination between the benign lesions, carcinoma in situ and invasive carcinoma.[35] Several metabolomics studies showed that Cho and Cho derivate compounds, designated as total Cho can be used as a biomarker for differentiating malignant breast tissue from normal tissue.[36,45,46] ^{1}H NMR spectroscopy was also used to identify the differences in the metabolic pattern of involved and non-involved axillary lymph nodes of breast cancer patients. The results depicted a higher level of glycerophosphocholine (GPC), PC and lactate (Lac) in involved axillary lymph nodes as compared to non-involved axillary lymph nodes showing the potential of NMR in the prediction of breast cancer patients.[47] Silva et al. has used urinary ^{1}H NMR based metabolomic approach to identify breast cancer (BC) specific metabolites that may be employed for diagnosis of BC. The metabolites such as creatine, glycine, trimethylamine N-oxide, and serine showed highest sensitivity and specificity and were to discriminate BC patients from control.[7] ^{1}H NMR spectroscopy was also employed to predict the plasma metabolites associated with long-term risk of developing breast cancer. The study revealed that women with higher fasting plasma levels of valine, lysine, arginine, glutamine, creatine, creatinine and glucose and lower plasma levels of lipoproteins, lipids, glycoproteins, acetone, glycerol derived compounds and unsaturated lipids had a higher risk of developing breast cancer.[20]

Prostate Cancer

Metabolomics has been proved to be a promising tool for elucidating the biochemical pathways effected by prostate cancer and for identification of new novel clinical biomarkers in biofluids.[48] High-resolution magic angle spinning ^{1}H spectroscopy was also used to determine the concentration of several prostate metabolites and compared between normal and cancer tissue.[36] Concentrations of total Cho, PC/GPC ratio, Lac, and alanine (Ala) were found to be higher in prostate cancer compared to healthy glandular and stromal tissues, while citrate (Cit) and polyamine concentrations were found to be significantly elevated in healthy glandular tissues in contrast to healthy stromal or and prostate cancer tissues.[36] Metabolic profiling of seminal fluid and prostatic secretion using ^{1}H NMR showed that Cit based prostate cancer detection surpass prostate specific antigen testing.[37] A ^{1}H NMR spectroscopic study demonstrated that absolute concentration of myo-inositol and spermine, in addition to citrate were highly predictive of prostate cancer.[49] Giskeodegard et al. reported the changes in levels of fatty acids, arginine and phospholipids in the blood plasma and serum of patients with prostate cancer and benign prostate hyperplasia using three analytical techniques, mass spectrometry, gas chromatography and NMR spectroscopy.[50] A ^{1}H NMR based spectroscopic profiling of filtered serum demonstrated that glycine, sarcosine, alanine, creatine, xanthine and hypoxanthine were able to distinguish abnormal prostate with respect to healthy control.[51]

Diabetes

Metabolomics study is ideal for studying metabolic disorders such as diabetes. NMR spectroscopy has been extensively employed for investigating metabolic alterations in type 1 and type 2 diabetes.[52,53] Salek et al. investigated the metabolic profile of urine in two rodent models with type 2 diabetes and human sufferers.[52] The study demonstrated metabolic similarities between all the three groups, including perturbation in tricarboxylic acid (TCA) cycle, nucleotide metabolism and methylamine metabolism.[52] Moreover, the results suggest that

metabolites such as N-methylnicotinamide and N-methyl-2-pyridone-5-carboxamide may serve as biomarker/s for monitoring type 2 diabetes progressions.[52] NMR based metabolomics was used to determine the concentration of metabolites in urine of diabetic and healthy Sardinian children.[53] It was reported that metabolites such p-cresol sulphate and phenylacetylglycine are responsible for the distinction between the diabetic and healthy children, suggesting that perturbation in gut microflora is associated with type 1 diabetes.[53] Bervoets et al. investigated the metabolic profile of plasma in children and adolescents with type 1 diabetes mellitus. The study showed that in addition to higher concentration of glucose, lipids (triglycerides, phospholipids and cholinated phospholipids) and concentrations of the amino acids serine, tryptophan and cysteine were found to be decreased in T1DM patients as compared to controls. Thus the metabolic profiling of plasma ^{1}H NMR spectroscopy discriminates between T1DM patients and control.[54] In one of the recent study by Coco et al identified the metabolic signature of serum associated with T2DM stages. The metabolic perturbation suggested branched chain amino acids (BCAAs) were significantly compromised in T2DM patients while perturbation in gluconeogenic amino acids other than BCAAs suggest both early and advanced T2DM stages.[55]

Coronary artery disease (CAD)

^{1}H NMR spectroscopy in conjunction with multivariate analysis was utilized for the diagnosis and for determining the severity of coronary heart disease.[56] The study documented that multivariate analysis of ^{1}H NMR spectra of blood serum could predict the angiographically defined CAD with > 90% accuracy and specificity.[57] Later studies have shown that the detection of coronary artery disease by ^{1}H NMR spectroscopy was affected by the confounding factors such as genders, hormonal status, statin and diet and hence, reduce the accuracy and specificity of prediction.[57] A ^{1}H NMR spectroscopic study investigated the metabolic profile of blood plasma of patients with CAD and controls to identify biomarkers for differentiating patients from controls and for differentiation of patients with single vessel disease, double vessel disease and triple vessel disease. Metabolites namely, Ala, Isoleucine/Leucine/valine (Ile/Leu/Val) and low density lipoprotein/very low density lipoprotein (LDL/VLDL) were suggested as putative biomarkers to demarcate between controls and patients with single vessel disease, double vessel disease and triple vessel disease.[58] The study demonstrates the role of metabolomics in differentiating between patients with different outcome after an acute myocardial infarction. Metabolomics depicted differential clustering between two cohorts with training set showing sensitivity of 76.9%, specificity of 79.5%, accuracy of 78.2% and AUC of 0.859. The results were reproduce in the validation set with sensitivity, specificity and accuracy of 72.6 % in each respectively.[9] One of the recent study has investigated the sex associated differences in the ^{1}H NMR metabolic profile of patients with suspected CAD in addition to lipoproteins. Apart from lipoproteins, higher level of glucose and lactate were found in women in deproteinized serum.[59]

Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) include a group of inflammatory diseases that affect colon and the small intestine.[60,61] The two most common type of IBD are Crohn's disease (CD) and ulcerative colitis (UC). High-resolution ^{1}H NMR spectroscopy combined with multivariate analysis was used for the characterization of serum, plasma, and urine to differentiate between healthy subjects and patients with CD and UC.[62] The results suggested that metabolic profiling of plasma, serum and urine can be used to differentiate between IBD patients and healthy controls.[62] Marchesi and co-workers investigated the metabolic profile of faecal extracts obtained from patients with CD and UC and healthy subjects using NMR based metabolomics.[63] The results illustrated that metabolites such as butyrate, Ace, methylamine, and trimethylamine were present in a lower concentration in faeces of both CD and UC patients as compared to controls. ^{1}H NMR spectroscopy has also been used for metabolic profiling of colonic mucosa in patients with IBD in order to identify a possible biomarker that could differentiate between UC and CD.[64] Formate (For) was found to be a likely biomarker for differentiation of active phase of UC from that of the active phase of CD.[64] Bjerrum et al investigated the metabolic profile of faecal extracts obtained from healthy subjects and patients with active or inactive CD and UC and using NMR based metabolomics. The results illustrated that metabolites such as amino acids, micro-biota related fatty acids and lactate suggestive of inflammation driven changes in the metabolic profiles related to malabsorption and dysbiosis.[65] ^{1}H NMR spectroscopy has also been used for metabolic profiling of urine in children with IBD with respect to control. Metabolic differences include central energy metabolism, amino acid, and gut microbial metabolic pathways. The analysis described that combined urinary urea and phenylacetylglutamine—two readouts of nitrogen metabolism—may be relevant to monitor metabolic status in the course of disease.[66]

Celiac Disease

Bertini et al. have reported metabolic profile of urine and serum samples of celiac disease (CeD) patients prior to treatment, patients on gluten free

diet (GFD), and healthy controls using ^{1}H NMR spectroscopy.[67] Sera of CeD patients were shown to have lower levels of several metabolites such as amino acids, lipids, Pyr and Cho, and by increased levels of Glc and β-hydroxybutyrate (β-OHB), while in urine altered level of acetoacetate (AcAc), indoxyl sulphate (IS), meta-hydroxyphenyl- propionic acid and PAG was observed. The most remarkable finding of this study was that the metabolic pattern including levels of Glc and β-OHB of CeD patients normalized after the 12 months of strict GFD.[67]

The same group also reported the metabolic profile of the potential CeD patients using NMR spectroscopy.[68] Strikingly, the metabolic pattern of sera of potential CeD patients was very much closer to that of CeD patients, despite no intestinal damage.[68] Further, Rezaei-Tavirani et al. employed ^{1}H NMR spectroscopy in conjunction with chemometric analysis to evaluate the serum samples of CeD patients, patients on GFD and healthy controls.[69] It was observed that the metabolites such as lipid, Lac and Cho can be used as biomarker/s to demarcate between patients with CeD, patients on GFD and healthy controls.[69] Also in another study by Fathi et al. also reported significantly reduced level of Lac, Val and lipids in the serum of CeD patients in comparison to healthy controls using NMR spectroscopy.[70]

We have documented the abnormalities in various metabolic pathways in the small intestinal mucosa of patients with CeD.[71] Recently our group reported the metabolic profile of small intestinal mucosa, blood plasma and urine and demonstrated 9 altered metabolic pathways contributing to the pathogenesis of CeD. Classification model was calculated using a combination of differentiating metabolites of blood plasma and urine with a sensitivity of 97.7%, specificity of 93.3%. The predictive accuracy was 95.1% for the model.[11]

Conclusion

This review highlights the importance and application of NMR based metabolomics in disease diagnosis and progression. Metabolomics is a rapidly expanding new omics science and encompasses a useful approach for identification of disease-related metabolites also known as biomarkers in biofluids or tissue. The various pre and post processing steps, along with validation of biomarkers and metabolic pathway analysis provides a promising and better insight into the disease mechanism. With improvement in multivariate statistical analysis method NMR based metabolomics may serve as sensitive and appropriate technique for early disease diagnosis and also its therapeutic approach.

References

1. Goodacre R. Metabolomics – the way forward. Metabolomics 2005; 1: 1-2.
2. Lindon J, Nicholson J, Holmes E. The Handbook of Metabonomics and Metabolomics. 1st edition, 2006, Elsevier.
3. Want EJ, Nordström A, Morita H, Siuzdak G. From exogenous to endogenous: the inevitable imprint of mass spectrometry in metabolomics. J Proteome Res. 2007; 6(2): 459-68.
4. Nicholson JK, Wilson ID. Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. Nat Rev Drug Discov. 2003; 2(8): 668-76.
5. Patti GJ, Yanes O, Siuzdak G. Innovation: Metabolomics: the apogee of the omics trilogy. Nat Rev Mol Cell Biol. 2012; 13(4): 263-9.
6. Kaddurah-Daouk R, Kristal BS, Weinshilboum RM. Metabolomics: a global biochemical approach to drug response and disease. Annu Rev Pharmacol and Toxicol. 2008; 48(1): 653-83.
7. Silva C, Perestrelo R, Silva P, Tomás H , Câmara JS. Breast Cancer Metabolomics: From Analytical Platforms to Multivariate Data Analysis. A Review. Metabolites 2019; 9(5): 102.
8. Lima AR, Pinto J, Bastos ML, Carvalho M, Pinho PG. NMR-based metabolomics studies of human prostate cancer tissue. Metabolomics 2018; 14(7): 88.
9. Vignoli A, Tenori L, Giusti B,Takis PG, Valente S, Carrabba N, et al. NMR-based Metabolomics Identifies Patients at High Risk of Death within Two Years after Acute Myocardial Infarction in the AMI-Florence II Cohort. BMC Med. 2019; 17(1): 3.
10. Probert F, Walsh A, Jagielowicz M, Yeo T, Claridge TDW, Simmons A, et al. Plasma Nuclear Magnetic Resonance Metabolomics Discriminates Between High and Low Endoscopic Activity and Predicts Progression in a Prospective Cohort of Patients With Ulcerative Colitis. J Crohn's and Colitis 2018; 12(11): 1326–1337.
11. Upadhyay D, Singh A, Das P, Mehtab J, Dattagupta S, Ahuja V, et al. Abnormalities in metabolic pathways in Celiac disease investigated by the metabolic profiling of small intestinal mucosa, blood plasma and urine by NMR spectroscopy. NMR Biomed. 2020; 33(8): e4305.
12. Gromski PS, Muhamadali H, Ellis DI, Xu Y, Correa E, Turner ML, et al. A tutorial review: Metabolomics and partial least squares-discriminant analysis—A marriage of convenience or a shotgun wedding. Anal Chim Acta. 2015; 879: 10–23.
13. Van den Berg RA, Hoefsloot HC, Westerhuis JA, Smilde AK, van der Werf MJ. Centering, scaling, and transformations: Improving the biological information content of metabolomics data. BMC Genomics 2006; 7: 142.

14. Sysi-Aho M, Katajamaa M, Yetukuri L, Oreši ˇc M. Normalization method for metabolomics data using optimal selection of multiple internal standards. BMC Bioinformatics 2007; 8: 93.

15. Kohl SM, Klein MS, Hochrein J, Oefner PJ, Spang R, Gronwald W. State-of-the art data normalization methods improve NMR-based metabolomic analysis. Metabolomics 2012; 8(supp 1): 146–160.

16. Jobard E, Pontoizeau C, Blaise BJ, Bachelot T, Elena-Herrmann B, Trédan O. A serum nuclear magnetic resonance-based metabolomic signature of advanced metastatic human breast cancer. Cancer Lett. 2014; 343(1): 33–41.

17. Cífková E, Lísa M, Hrstka R, Vrána D, Gat ˇek J, Melichar B, et al. Correlation of lipidomic composition of cell lines and tissues of breast cancer patients using hydrophilic interaction liquid chromatography/electrospray ionization mass spectrometry and multivariate data analysis. Rapid Commun Mass Spectrom. 2017; 31(3): 253–263.

18. Porto-Figueira, P, Pereira JAM, Câmara JS. Exploring the potential of needle trap microextraction combined with chromatographic and statistical data to discriminate different types of cancer based on urinary volatomic biosignature. Anal Chim Acta. 2018; 1023: 53–63.

19. Martinez-Lozano Sinues P, Landoni E, Miceli R, Dibari VF, Dugo M, Agresti R, et al. Secondary electrospray ionization-mass spectrometry and a novel statistical bioinformatic approach identifies a cancer-related profile in exhaled breath of breast cancer patients: A pilot study. J Breath Res. 2015; 9(3): 31001.

20. Lécuyer L, Victor Bala A, Deschasaux M, Bouchemal N, Nawfal Triba M, Vasson MP, et al. NMR metabolomic signatures reveal predictive plasma metabolites associated with long-term risk of developing breast cancer. Int J Epidemiol. 2018; 47(2): 484–494.

21. Yi L, Dong N, Yun Y, Deng B, Ren D, Liu S, et al. Chemometric methods in data processing of mass spectrometry-based metabolomics: A review. Anal Chim Acta. 2016; 914: 17–34.

22. Brauer MJ, Yuan J, Bennett BD, Lu W, Kimball E, Botstein D, et al. Conservation of the metabolomic response to starvation across two divergent microbes. Proc Natl Acad Sci. U.S.A. 2006; 103: 19302–19307.

23. Sreekumar A, Poisson LM, Rajendiran TM, Khan AP, Cao Q, Yu J, et al. Metabolomic profiles delineate potential role for sarcosine in prostate cancer progression. Nature 2009; 457(7231): 910–914.

24. Trygg J, Wold S. Orthogonal projections to latent structures (O-PLS). J chemom. 2002; 16 (3): 119-128.

25. Xia J, Broadhurst DI, Wilson M, Wishart DS. Translational biomarker discovery in clinical metabolomics: an introductory tutorial. Metabolomics 2013; 9(2): 280–299.

26. Huang S, Chong N, Lewis NE, Jia W, Xie G, Garmire LX. Novel personalized pathway-based metabolomics models reveal key metabolic pathways for breast cancer diagnosis. Genome Med. 2016; 8(1): 34.

27. Williams MD, Zhang X, Park JJ, Siems WF, Gang DR, Resar LMS, et al. Characterizing metabolic changes in human colorectal cancer. Anal Bioanal Chem. 2015; 407(16): 4581–4595.

28. Gonzalez-Dom, Inguez R, Sayago A, Fernandez-Recamales ´ A. Metabolomics in Alzheimer's disease: the need of complementary analytical ´platforms for the identification of biomarkers to unravel the underlying pathology. J Chromatogr B. 2017; 1071: 75–92

29. Murgia F , Muroni A, Puligheddu M, Polizzi L, Barberini L, Orofino G, et al. Metabolomics as a tool for the characterization of drug-resistant epilepsy. Front Neurol. 2017; 8(459): 1–9.

30. Tao Y, Chen X, Cai H, Li W, Cai B, Chai C, et al. Untargeted serum metabolomics reveals Fu-Zhu-Jiang-Tang tablet and its optimal combination improve an impaired glucose and lipid metabolism in type II diabetic rats. J Chromatogr B. 2017; 1040: 222–232.

31. Cuykx M, Claes L, Rodrigues RM, Vanhaecke T, Covaci A. Metabolomics profiling of steatosis progression in HepaRGR cells using sodium valproate. Toxicol Lett. 2018; 286: 22–30.

32. Wang Z, Liu JQ, Xu JD, Zhu H, Kong M, Zhang GH, et al. UPLC/ESI-QTOF-MS-based metabolomics survey on the toxicity of triptolide and detoxication of licorice. Chinese J Nat Med. 2017; 15(6): 474–480.

33. La Barbera G, Capriotti AL, Cavaliere C, Montone CM, Piovesana S, Samperi R, et al. Liquid chromatography–high resolution mass spectrometry for the analysis of phytochemicals in vegetal-derived food and beverages. Food Res Int. 2017; 100(Part 1): 28–52.

34. Mackinnon WB, Barry PA, Malycha PL, Gillett DJ, Russell P, Lean CL, et al. Fine-needle biopsy specimens of benign breast lesions distinguished from invasive cancer ex vivo with proton MR spectroscopy. Radiology 1997; 204(3): 661-6.

35. Swanson MG, Zektzer AS, Tabatabai ZL, Simko J, Jarso S, Keshari KR, et al. Quantitative analysis of prostate metabolites using ^{1}H HR-MAS spectroscopy. Magn Reson Med. 2006; 55(6): 1257-64.

36. Kline EE, Treat EG, Averna TA, Davis MS, Smith AY, Sillerud LO. Citrate concentrations in human seminal fluid and expressed prostatic fluid determined via ^{1}H nuclear magnetic resonance spectroscopy outperform prostate specific antigen in prostate cancer detection. J Urol. 2006; 176(5): 2274-9.

37. Kumar V, Dwivedi DK, Jagannathan NR. High-resolution NMR spectroscopy of human body fluids and tissues in relation to prostate cancer. NMR Biomed. 2014; 27(1): 80-9

38. Beger RD, Schnackenberg LK, Holland RD, Li D, Dragan Y. Metabonomic models of human pancreatic cancer using 1D proton NMR spectra of lipids in plasma. Metabolomics 2006; 29(3): 125-34.

39. Fang F, He X, Deng H, Chen Q, Lu J, Spraul M, et al. Discrimination of metabolic profiles of pancreatic cancer from chronic pancreatitis by high-resolution magic angle spinning ^{1}H nuclear magnetic resonance and principal components analysis. Cancer Sci. 2007; 98(11): 1678-82.

40. Hori S, Nishiumi S, Kobayashi K, Shinohara M, Hatakeyama Y, Kotani Y, et al. A metabolomic approach to lung cancer. Lung Cancer 2011; 74(2): 284-92.

41. Spratlin JL, Serkova NJ, Eckhardt SG. Clinical applications of metabolomics in oncology: a review. Clin Cancer Res. 2009; 15(2): 431-40.

42. Griffin JL, Shockcor JP. Metabolic profiles of cancer cells. Nat Rev Cancer 2004; 4(7): 551-61.

43. Mackinnon WB, Barry PA, Malycha PL, Gillett DJ, Russell P, Lean CL, et al. Fine-needle biopsy specimens of benign breast lesions distinguished from invasive cancer ex vivo with proton MR spectroscopy. Radiology 1997; 204(3): 661-6.

44. Nicholson JK, Lindon JC, Holmes E. 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. Xenobiotica 1999; 29(11): 1181-9.

45. Cheng LL, Chang IW, Smith BL, Gonzalez RG. Evaluating human breast ductal carcinomas with high-resolution magic-angle spinning proton magnetic resonance spectroscopy. J Magn Reson. 1998; 135(1): 194-202.

46. Li M, Song Y, Cho N, Chang JM, Koo HR, Yi A, et al. An HR-MAS MR metabolomics study on breast tissues obtained with core needle biopsy. PLoS One 2011; 6(10): e25563.

47. Sharma U, Mehta A, Seenu V, Jagannathan NR. Biochemical characterization of metastatic lymph nodes of breast cancer patients by in vitro ^{1}H magnetic resonance spectroscopy: a pilot study. Magn Reson Imaging 2004; 22(5): 697-706.

48. Gómez-Cebrián N, Rojas-Benedicto A, Albors-Vaquer A, López-Guerrero JA, Pineda-Lucena A, Puchades-Carrasco L. Metabolomics Contributions to the Discovery of Prostate Cancer Biomarkers. Metabolites 2019; 9(3): 48

49. Serkova NJ, Gamito EJ, Jones RH, O'Donnell C, Brown JL, Green S, et al. The metabolites citrate, myo-inositol, and spermine are potential age-independent markers of prostate cancer in human expressed prostatic secretions. Prostate 2008; 68(6): 620-8.

50. Giskeødegård GF, Hansen AF, Bertilsson H, Gonzalez SV, Kristiansen KA, Bruheim P, et al. Metabolic markers in blood can separate prostate cancer from benign prostatic hyperplasia. Br J Cancer 2015; 113: 1712-9.

51. Kumar D, Gupta A, Mandhani A, Sankhwar SN. NMR spectroscopy of filtered serum of prostate cancer: A new frontier in metabolomics. Prostate 2016; 76(12): 1106-19.

52. Salek RM, Maguire ML, Bentley E, Rubtsov DV, Hough T, Cheeseman M, et al. A metabolomic comparison of urinary changes in type 2 diabetes in mouse, rat, and human. Physiol Genomics 2007; 29(2): 99-108

53. Culeddu N, Chessa M, Porcu MC, Fresu P, Tonolo G, Virgilio G, et al. NMR-based metabolomic study of type 1 diabetes. Metabolomics 2012; 8(6): 1162-69.

54. Bervoets L, Massa G, Guedens W, Louis E, Noben JP, Adriaensens P. Metabolic profiling of type 1 diabetes mellitus in children and adolescents: a case-control study. Diabetol Metab Syndr. 2017; 9: 48

55. Coco L Del, Vergara D, Matteis S De, Mensà E, Sabbatinelli J, Prattichizzo F, et al. NMR-Based Metabolomic Approach Tracks Potential Serum Biomarkers of Disease Progression in Patients with Type 2 Diabetes Mellitus. J clin Med. 2019; 8(5): 720.

56. Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, et al. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ^{1}H-NMR-based metabonomics. Nat Med. 2002; 8(12): 1439-44.

57. Kirschenlohr HL, Griffin JL, Clarke SC, Rhydwen R, Grace AA, Schofield PM, et al. Proton NMR analysis of plasma is a weak predictor of coronary artery disease. Nat Med. 2006; 12(6): 705-10.

58. Malik A, Sharma U, Lakshmy R, Narang R, Jagannathan NR. Biochemical characterization of blood plasma of coronary artery disease patients by in vitro high-resolution proton NMR spectroscopy. J Biosci. 2015; 40(1): 31-9.

59. Barba I, Andrés M, Picón I, Aguade-Bruix S, Garcia-Dorado D. Sex differences in the ^{1}H NMR metabolic profile of serum in cardiovascular risk patients. Scientific Reports 2019; 9(1): 2380.

60. Ahmed I, Roy BC, Khan SA, Septer S, Umar S. Microbiome, Metabolome and Inflammatory Bowel Disease. Microorganisms 2016; 4(2): 20.

61. Baumgart DC, Carding SR. Inflammatory bowel disease: cause and immunobiology. Lancet 2007; 369(9573): 1627-40.

62. Schicho R, Shaykhutdinov R, Ngo J, Nazyrova A, Schneider C, Panaccione R, et al. Quantitative metabolomic profiling of serum, plasma, and urine by (1)H NMR spectroscopy discriminates between patients with inflammatory bowel disease and healthy individuals. J Proteome Res. 2012; 11(6): 3344-57.

63. Marchesi JR, Holmes E, Khan F, Kochhar S, Scanlan P, Shanahan F, et al. Rapid and noninvasive metabonomic characterization of inflammatory bowel disease. J Proteome Res. 2007; 6(2): 546-51.

64. Balasubramanian K, Kumar S, Singh RR, Sharma U, Ahuja V, Makharia GK, et al. Metabolism of the colonic mucosa in patients with inflammatory bowel diseases: an in vitro proton magnetic resonance spectroscopy study. Magn Reson Imaging 2009; 27(1): 79-86.

65. Bjerrum JT, Wang Y, Hao F, Coskun M, Ludwig C, Günther U, et al. Metabonomics of human fecal extracts characterize ulcerative colitis, Crohn's disease and healthy individuals. Metabolomics 2015;11:122-133.

66. Martin FP, Ezri J, Cominetti O, Silva Da L, Kussmann M, Godin JP, et al. Urinary Metabolic Phenotyping Reveals Differences in the Metabolic Status of Healthy and Inflammatory Bowel Disease (IBD) Children in Relation to Growth and Disease Activity. Int J Mol Sci. 2016; 17(8): 1310.

67. Bertini I, Calabrò A, De Carli V, Luchinat C, Nepi S, Porfirio B, et al. The metabonomics signature of celiac disease. J Proteome Res. 2009; 8(1): 170-7.

68. Bernini P, Bertini I, Calabrò A, la Marca G, Lami G, Luchinat C, et al. Are patients with potential celiac disease really potential? The answer of metabonomics. J Proteome Res. 2011; 10(2): 714-21.

69. Rezaei-Tavirani M, Fathi F, Darvizeh F, Zali MR, Rostami Nejad M, Rostami K, et al. Advantage of Applying OSC to (1)H NMR-Based Metabonomic Data of Celiac Disease. Int J Endocrinol Metab. 2012; 10(3): 548-52.

70. Fathi F, Ektefa F, Arefi Oskouie A, Rostami K, Rezaei-Tavirani M, Mohammad Alizadeh AH, et al. NMR based metabonomics study on celiac disease in the blood serum. Gastroenterol Hepatol Bed Bench. 2013; 6(4): 190-4.

71. Sharma U, Upadhyay D, Mewar S, Mishra A, Das P, Datta Gupta S, et al. Metabolic abnormalities of gastrointestinal mucosa in celiac disease: an in-vitro proton NMR spectroscopy study. J Gastroenterol Hepatol. 2015; 30(10): 1492-8.